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Hexadecane Mineralization in Oxygen-Controlled Sediment-Seawater Cultivations with Autochthonous Microorganisms

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Laboratory studies investigated the influence of dissolved oxygen tension (DOT) on microbial degradation of hexadecane in cultures with sediment-seawater suspensions. With a fermentor system, it was possible to adjust and regulate different oxic conditions (DOTs between 0.4 and 80% of oxygen saturation) as well as anoxia. The effects of DOT reduction on the amount and rate of hexadecane degraded and on the degree of mineralization and on the production of biomass were investigated. When the DOT was reduced from 80% to 5%, no dependence of the investigated parameters on the oxygen concentration was found. The amount of hexadecane degraded was constant, with an average value of 86% of the initially applied amount. The degradation rate was constant even down to 1% DOT, with an average value of 0.15 mg of hexadecane per g of sediment per h (16.2 mg liter^{-1} h^{-1}). The mean degree of mineralization was 70% of the initially applied hexadecane, and biomass production reached a value of about 1.5 g per g of hexadecane consumed. A significant influence on the degradation process was detected only with DOTs below 1%. The degree of mineralization and the amount of degraded hexadecane decreased, whereas the degradation rate was still unaffected. Under anoxic conditions, no hexadecane degradation occurred within 190 h. The fact that the hexadecane biodegradation rate was constant down to at least 0.4% DOT shows that the actual oxygen concentration is of minor importance as long as the oxygen supply is high enough to guarantee the oxygen-dependent degradation step.

Petroleum hydrocarbons are well suited to biological treatment. Consequently, in situ remediation has been applied most frequently to this type of contamination. In oil-polluted marine intertidal sediments, in which mechanical removal of oil and other reclamation technologies are not feasible, bioremediation is the only alternative. The degree and rate of hydrocarbon biodegradation are influenced by a variety of parameters, such as temperature, nutrient content, pH, and availability of oxygen (4, 6, 11, 12, 17, 18, 22, 27, 37). In sediment and soil, the oxygen content decreases with decreasing grain size and increasing moisture content. Especially in muddy sediments, oxygen is often limited and penetrates only millimeters or centimeters (2, 20). To assess the effectiveness of microbial degradation of hydrocarbons and to support remediation of sediment, the influence of different oxygen concentrations has to be investigated. For these investigations the saturated hydrocarbon *n*-hexadecane is well suited as a model substance. Under aerobic conditions, oxygen in the form of O_2 is required for the initial degradation step (7, 10, 33, 35). Degradation of *n*-alkane to alkene by dehydrogenation under anaerobic conditions was suggested but is controversial because of the thermodynamic problems involved (3, 32). Anaerobic oxidation of hexadecane by a sulfate-reducing bacterium was described recently (1). The anaerobic degradation rates reported were very slow and far beyond the time scale of biotechnical restoration or microbial remediation strategies.

The objective of this study was to evaluate the influence of different dissolved oxygen tensions (DOTs) on microbial mineralization of hexadecane. Laboratory studies were conducted with artificially contaminated marine sediment in a seawater suspension (11% [vol/vol] sediment) containing the indigenous microbial community. The dissolved oxygen concentration was kept constant between 80 and 0.4% DOT. In this paper we describe the degradation and mineralization rates of hexadecane and the production of biomass as functions of the DOT.

MATERIALS AND METHODS

Sediment and sediment treatment. The sediment used in this study was taken from the German Wadden Sea in the Jadebusen Bay. Grain size was analyzed by the Senckenberg Institute, Wilhelmshaven, Germany. The grain size distribution was 14.8% clay, 44.6% silt, and 40.7% sand. Before the sediment was used in the experiments, it was air dried, sieved, and homogenized. The sediment was treated with hexadecane (10 ml per 100 g of sediment) and a larger amount of pentane (sufficient to form a slurry with the dry sediment material). After thorough mixing, the pentane was evaporated exhaustively in a rotation evaporator (60°C) and the sediment was dried for 12 h at 60°C. Gas chromatographic analysis showed a hexadecane concentration of 61.2 g per kg of sediment. The coated sediment was stored in sterile bottles at 4°C.

Microorganisms and precultures. All cultures contained the autochthonous population of microorganisms from the intertidal sediment. In addition, the cultures were inoculated with hexadecane-adapted microorganisms. To obtain this adapted population, 50 g of wet sediment (taken from a basin in which mudflat conditions are simulated) was mixed with 56.4 ml of artifical seawater (23) containing 1 ml of hexadecane and incubated for 48 h at 100 to 120 rpm on a gyratory shaker at 20°C. The microorganisms in the supernatant of this preculture were further incubated in 106 ml of artificial seawater (without sediment but supplemented with vitamins, mineral salts, ammonia or nitrate, and phosphate) containing

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1 ml of hexadecane. The incubation conditions were identical. The microorganisms from this incubation were used as the inoculum for the fermentations.

Determination of cell concentrations. To determine the concentration of cells in the sediment suspension, 1 ml of sediment-seawater suspension was mixed with 4.0 ml of KH_2PO_4 (0.1 mol liter^{-1}) containing 0.5% (vol/vol) Tween 80. The dilution was sonified with a Branson sonifier three times for 30 s with a break of 30 s (50 W, 50 Hz). After 30 s of sedimentation, aliquots of the supernatant were taken; the cell number was determined by counting in a hemacytometer (Thoma) at a depth of 0.02 mm. To determine the cell concentration in the absence of sediment, the samples were sonified as described above but without KH_2PO_4.

Chemicals. All chemicals used were of the highest purity available and obtained from Merck (Darmstadt), Fluka (Ulm), Sigma (Deisenhofen), and Riedel de Haen (Seelze). Helium-4.6, nitrogen-5.0, and the control gas (98% N_2, 2% O_2) for the equilibration of the gas analyzer and the oxygen electrode were obtained from Messer Griesheim (Duisburg).

Fermentation system. The fermentations were performed in a stirred and aerated reactor with a working volume of 2.1 liters (Braun Diessel Biotech GmbH, Melsungen, Germany). The reactor was equipped with registration and control units for pH, DOT, and temperature. Cultures were aerated by means of a complex gas inlet system permitting two independent gas flow rates that could be regulated separately. This system consisted of a modified proportional action controller of the type E 565 (Metrohm, Herisau, Switzerland) and Hi-Tec mass flow meters (Bronkhorst B. V., Ruurlo, The Netherlands). The regulation of the gas flow rates depended on the deviation of the actual DOT from the fixed nominal value (26).

Culture conditions. The fermentations were performed with 11% (vol/vol) dried sediment in artificial seawater (23). Hexadecane-coated sediment was mixed with uncoated sediment, resulting in an initial hexadecane concentration of 1.2 g per liter of sediment-seawater suspension. Nitrogen (nitrate or ammonia) and phosphate were added separately at a concentration recommended by Gibbs (16): 60 mg of N and 6 mg of P for the degradation of 1 g of hydrocarbon. Previous investigations had confirmed that these concentrations are high enough to avoid N and P limitation (18). In the range of 80 to 5% DOT, $NaNO_3$ was used as the nitrogen source. In the range of 0 to 5% DOT, it was replaced by $(NH_4)_2SO_4$ to avoid denitrifying hexadecane degradation. Vitamins specified by Schlegel (34) and mineral salts specified by the Deutsche Sammlung von Mikroorganismen (13) were added as supplements. The concentration of inoculated hexadecane-adapted microorganisms was 5×10^7 cells per ml. The agitation (600 rpm), temperature (20°C), and nitrogen gas flow (50 or 100 ml min^{-1}) were kept constant. The pH was adjusted to 7.3. The DOT was adjusted to different values (0.0, 0.4, 1.0, 5.0, 10.0, 20.0, 40.0, and 80.0%) and kept constant during each cultivation.

Aerobic reference fermentations with sterile sediment were performed to ensure that all hexadecane losses were due to microbial degradation and not to other effects like stripping.

Gas analysis. The amount of air in the gas influent was measured on line (gas flow meters and controllers, mass flow control instrument type MFC F-201 A Hi-Tec; Bronkhorst), and the percentages of O_2 and CO_2 were calculated. The CO_2 concentration of the gas effluent was recorded on line by an infrared detector (Unor 6N; Maihak, Hamburg, Germany). The O_2 concentration in the gas effluent was analyzed in gas samples (40 ml) taken with a gas-proof syringe and analyzed in a Shimadzu GC 8A gas chromatograph equipped with a heat conductivity detector and a capillary column packed with Propac QS (100/120 MESH). The carrier gas was helium-4.6, and the gas flow rate was 40 ml min^{-1}. The temperature of injector and detector was 150°C, and the column temperature was 50°C. The injected sample volume was 0.25 ml. Integration was performed with a Shimadzu Chromatopac C-E1B integrator.

***n*-Hexadecane determination.** Hexadecane concentrations were determined by gas chromatography after extraction with *n*-hexane. A sample (10 ml) taken from the sediment suspension was mixed with an equal volume of hexane containing *n*-tetradecane as an internal standard, vigorously shaken, and centrifuged. Two milliliters of this supernatant was filled up to 10 ml with hexane and analyzed in a gas chromatograph (Perkin-Elmer 8310) under the following conditions: column packed with Chromosorb W-HP (80/100, 3% OV 101); carrier gas, nitrogen-5.0; gas flow rate, 38 ml min^{-1}; injector temperature, 270°C; flame ionization detector temperature, 290°C; oven temperature, 120 to 140°C with a ramp rate of 10°C min^{-1}, 140 to 270°C with a ramp rate of 30°C min^{-1}, and then 1 min at 270°C. The injection volume was 1 μl.

Protein determination. A variety of methods for determining biomass production of microorganisms in cultures with hydrocarbon as a substrate are discussed in the literature. Comparison of several of these methods indicates that none produces satisfactory results (14, 21, 25, 29, 31, 36). For the present investigations, the determination of the total protein content of the suspension was considered to be the most appropriate method. Samples (1 to 2 ml) of the sediment suspension were centrifuged, and the pellets were treated as described by Oberbremer and Müller-Hurtig (30). To test the recovery efficiency of protein in the sediment suspension, serum albumin and/or defined amounts of mixed bacterial populations were added. After the cultures were mixed and incubated for several hours (20°C, 100 rpm), the recovery efficiency ranged between 90 and 110%. The protein concentration was determined in pretreated pellets as described by Bradford (9).

Calculation of oxygen consumption and carbon dioxide production. The volumetric oxygen consumption (grams of oxygen consumed per liter of culture volume) and the volumetric carbon dioxide production (grams of carbon dioxide produced per liter of culture volume) were calculated from the gas inflow and outflow rates and from the concentrations of oxygen and carbon dioxide in the gas inflow and outflow, respectively.

RESULTS

Amount of degraded hexadecane and degradation rate. The dependence of hexadecane degradation on different DOTs was investigated in batch fermentations with sediment-seawater suspensions and mixed autochthonous populations of microorganisms of the intertidal sediment. The adjusted oxygen tensions ranged from 80 to 0% DOT. These values are relative to the maximum concentration of oxygen (100% DOT) that can be dissolved when air is added to the sediment-seawater suspension. The adjusted value was kept constant during each fermentation. The intended initial concentration of hexadecane was 1.2 g liter^{-1}. The concentration determined in the sediment suspension at the beginning of the cultures ranged from 1.1 to 1.5 g liter^{-1} (because of dilution of the hexadecane-coated sediment with uncoated

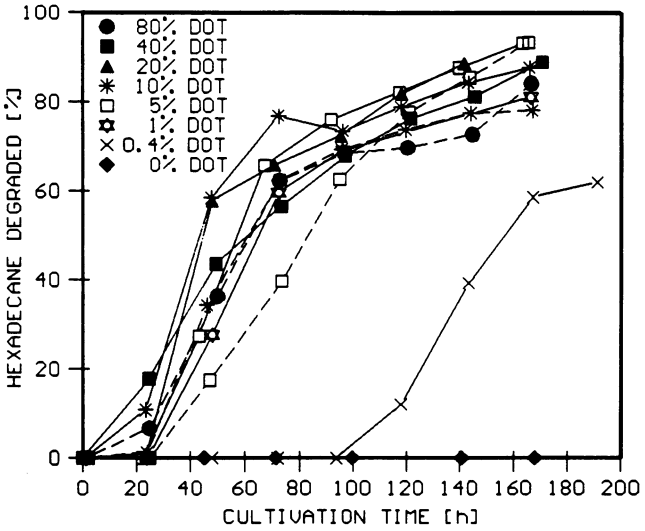

FIG. 1. Degradation of hexadecane (percentage of the initial concentration of hexadecane) in cultures with sediment-seawater suspensions at different DOTs.

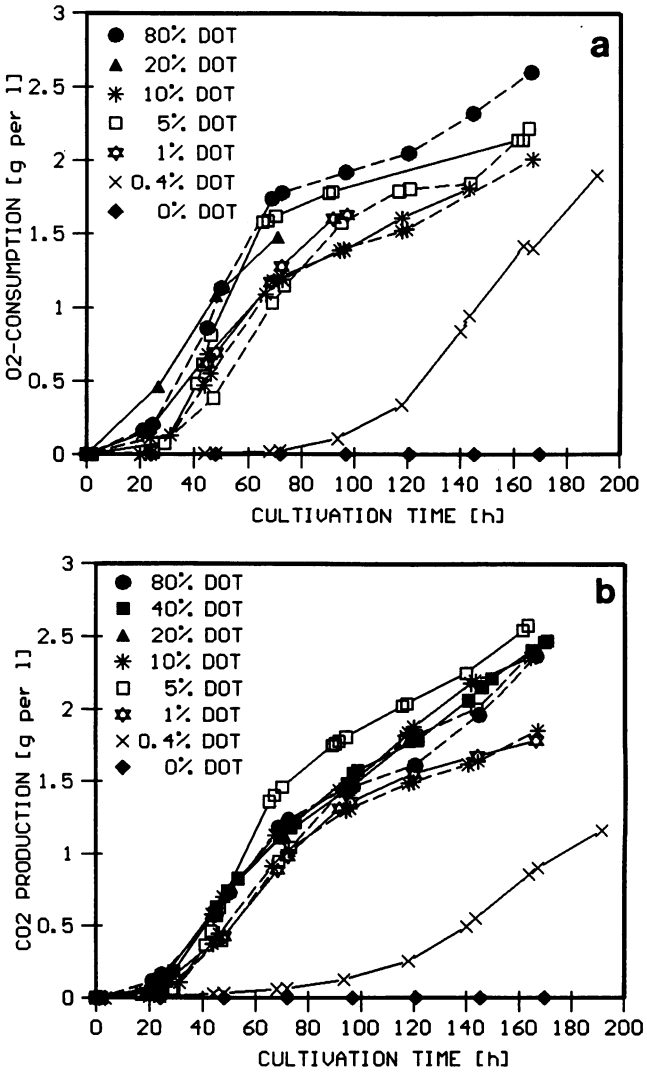

FIG. 2. O_2 consumption (a) and CO_2 production (b) during microbial degradation of hexadecane in cultures with sediment-seawater suspensions at different DOT values.

sediment). The actual hexadecane concentration of each culture was set to 100%.

There was no difference in the hexadecane degradation capacities of cultures with various DOTs in the range of 1 to 80% (Fig. 1). After 7 days of incubation, the final concentration of hexadecane ranged from 0.1 to 0.3 g liter^{-1}. That means that 78 to 93% (mean value, 86%) of the hexadecane quantity initially provided was degraded. An influence on the degree of degradation was found only with a DOT of 0.4%, the final concentration was 0.5 g liter^{-1}, and 62% of the originally applied hexadecane was degraded within the incubation time. No degradation was observed under anaerobic conditions.

The expected corresponding influence of the DOT on the hexadecane degradation rate was not detected. In the range of 0.4 to 80% DOT, a constant degradation rate of 16.2 mg liter^{-1} h^{-1} was obtained. Calculated with respect to the dry weight of the sediment (11%, vol/vol), the maximum degradation rate was 3.6 g of hexadecane per kg of sediment per day.

Another effect of low DOT (0.4%) was the noticeably expanded lag phase of more than 100 h, compared with lag phases of about 20 to 30 h for DOTs of 1 to 80%.

Production of biomass. The protein concentration of the original sediment suspension containing indigenous microorganisms was 0.19 g liter^{-1} (1.8 mg per g of dry sediment). Adding the inoculum led to a protein concentration of 0.23 g liter^{-1} (2.1 mg per g of dry sediment) and an average cell concentration of 5×10^9 cells per ml. In hexadecane-containing anaerobic cultivations (0% DOT) and in oxic hexadecane-free cultivations, no further protein was produced. In cultures with hexadecane and oxygen supplies, the protein concentration showed the typical dependence on time expected for batch cultures. The growth phase with the maximum protein production rate fitted well with the phase of the maximum degradation rate of hexadecane (data not shown). No influence of the DOT on the maximum protein production rate could be found. There was little influence of the DOT on the total amount of protein produced. In the range of 5 to 80% DOT and with a DOT of 0.4%, a yield of about 0.3 g of protein per g of hexadecane consumed was obtained. This value was only higher at a DOT of 1%, which yielded about 0.6 g of protein per g of hexadecane consumed. Careful checking of the method indicated that this value was not caused by experimental artifacts.

Respiration metabolism. The use of fermentors allowed us to determine the volumetric oxygen consumption (grams per liter of suspension) and the carbon dioxide production (grams per liter) during growth. Oxygen consumption (Fig. 2a) and carbon dioxide production (Fig. 2b) were independent of the DOT within the range of 80 to 1.0%. An influence was found only at a DOT of 0.4%, in which case the oxygen consumption and the carbon dioxide production were reduced; the carbon dioxide production was affected considerably more than was oxygen consumption. Neither CO_2 production nor O_2 consumption was detected under anaerobic conditions (Fig. 2).

The oxygen consumption curves and CO_2 production curves of the cultures with 1 to 80% DOT have parallel slopes (Fig. 2), which means that hexadecane is oxidized at

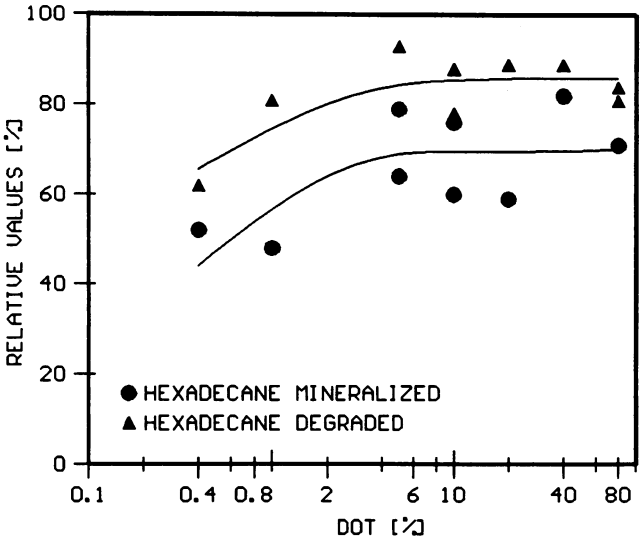

FIG. 3. Influence of DOTs between 0.4 and 80% on the degree of mineralization of consumed hexadecane (percentage of complete mineralization of hexadecane), and the extent of degraded hexadecane (percent referring to the initial hexadecane concentration).

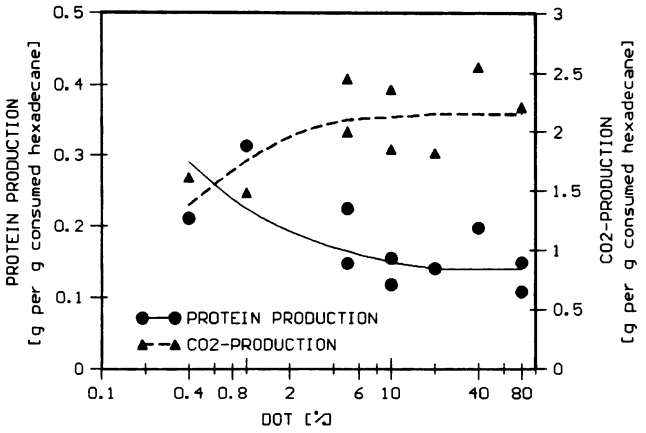

FIG. 4. Dependence of CO_2 production and protein production on the DOT.

a constant stoichiometric rate in these cultures. Control cultures were used to find out to what extent oxygen consumption and carbon dioxide production were due to indigenous carbon in the sediment or to added hexadecane. In the absence of hexadecane, the O_2 consumption ranged below 0.1 g liter^{-1} and the CO_2 production was below 0.04 g liter^{-1}. This negligible metabolic activity was probably due to the fact that the sediment had been exhausted by storage for several months.

Since the main metabolic activity of the cultures was due to hexadecane metabolism, the respiration coefficient (RQ) was a good measure for estimating the oxidative metabolism of hexadecane. The time course of the RQ during cultivation indicates two phases of substrate metabolism in cultures with 1 to 80% DOT. In the first cultivation phase (up to about 100 h), during which most hexadecane was consumed, the RQ was below the theoretical value of 0.65 mol mol^{-1} for complete hexadecane oxidation. It ranged between 0.5 and 0.6 mol mol^{-1}. In the later cultivation phase, the RQ exceeded the theoretical value and leveled off at about 0.75 mol mol^{-1} at the end of the cultivation time. In cultures with a DOT of 0.4%, the RQ was below the theoretical value during the whole cultivation time and ranged between 0.4 and 0.5 mol mol^{-1}.

Mineralization. If hexadecane is mineralized aerobically during catabolism without producing biomass or other metabolites, the cells need 24.5 mol of oxygen per mol of hexadecane and produce 16 mol of carbon dioxide and 17 mol of H_2O. Taking these data into account, the degree of mineralization of the consumed substrate was calculated. Since biomass production was found in all cultures except the anaerobic ones, complete oxidation of hexadecane to carbon dioxide was not expected. In the range of 5 to 80% DOT, a mean mineralization of 70% was found, whereas at 1% and 0.4% DOT the mineralization was only 50% (Fig. 3).

The calculated ratio between the amount of carbon dioxide produced per amount of hexadecane consumed (yield of CO_2, grams per gram) and the calculated yield of protein (grams of protein produced per gram of hexadecane consumed) indicated that more hexadecane is converted to biomass at DOTs below 5% than at DOTs of 5 to 80% (Fig. 4).

DISCUSSION

The aim of this investigation was to determine the degree and rate of hexadecane biodegradation and its dependence on different DOTs in seawater-sediment suspensions. Knowledge about the oxygen limitation of hydrocarbon biodegradation is important for strategies of remediation of spilled sediments. Previously published papers that deal with the dependence of the hexadecane degradation on the DOT do not contain data about degradation at DOTs near 0% (5, 15, 19, 26, 28).

To verify these culture conditions it was necessary to regulate and keep constant DOTs near 0%. This could only be done with a fermentor system. The crucial point was the development of a special mass flow regulation system consisting of two independent gas lines that allowed controlled mixing of a commercially available control gas (2% oxygen in nitrogen) with pure nitrogen.

These investigations showed that the total amount of degraded hexadecane was constant at 86% with DOTs of 1 to 80%. A decline of the hexadecane degradation to 62% occurred only at a DOT of less than 1%. This indicates that in sediment suspensions with a constant DOT of more than 1%, the oxygen concentration does not influence the amount of hydrocarbon degraded.

In this range of DOT, the hexadecane degradation rate was unaffected. Even when the DOT was lowered to 0.4%, the rate did not decline. Since in other studies different sediments and culture techniques were used, the results can hardly be compared (6). Nevertheless, a rough comparison gives a reasonable result: the mean degradation rate of 3.6 g of hexadecane per kg of sediment per day determined in this investigation is 20 times higher than that in previous batch cultures in Erlenmeyer flasks with an uncontrolled gas supply (19) and 7 times lower than those published by Oberbremer and Müller-Hurtig (30).

Considering the protein yield, which was constant at 0.3 g per g of hexadecane consumed (except the cultivation with 1% DOT), and considering the corresponding amount of hexadecane consumed, it is obvious that biomass production is not affected much by the actual oxygen concentration in

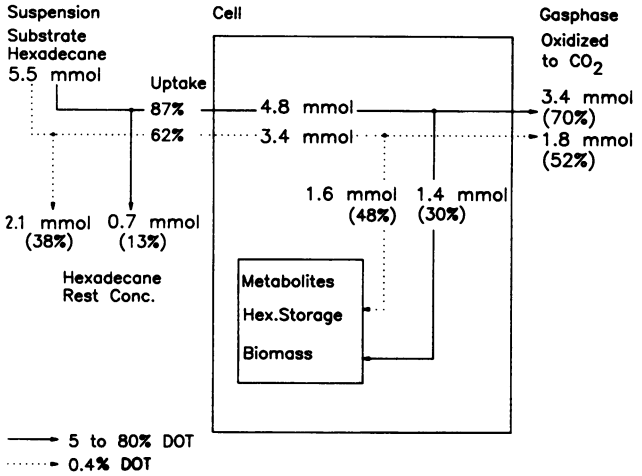

FIG. 5. Mass ratio of hexadecane uptake into cells to hexadecane mineralization at different DOT values in sediment-seawater suspensions with autochthonous microorganism populations.

the sediment suspension. With these data and with carbon dioxide production data, it is possible to calculate a mass balance for the hexadecane metabolism with respect to its dependence on the adjusted oxygen concentration. In the range of 5 to 80% DOT, the organisms consumed 87% (4.8 mmol) of the 5.5 mmol of hexadecane added. The residual 13% (0.7 mmol) remained in the suspension. With 0.4% DOT, 62% (3.4 mmol) of the offered 5.5 mmol of hexadecane was consumed and 38% (2.1 mmol) remained in the suspension. The hexadecane consumed was partly metabolized to biomass or accumulated in the cells and partly oxidized to carbon dioxide. In cultures with 5 to 80% DOT, 70% of the consumed hexadecane was mineralized to CO_2; in the case of 0.4% DOT, only 52% of the hexane was mineralized (Fig. 5).

The above-mentioned data do not explain why the oxidation of the consumed hexadecane does not depend on DOT. To determine this, other parameters like the oxygen affinity of the oxygen-dependent enzymes of hexadecane oxygenation have to be considered. In oxygen-saturated seawater with a salinity of 3% (in equilibrium with the atmosphere), the oxygen concentration is 210 μmol of O_2 per liter (20°C). If the DOT is adjusted to 1%, 2.1×10^{-6} mol of O_2 per liter is obtained. If hydroxylation is the rate-determining reaction in aerobic hexadecane degradation, the corresponding enzymes must be completely active at this oxygen concentration. Because of the problems involved in determinations of very small oxygen concentrations, little is known about the oxygen dependence (Michaelis constant) of the oxygenase enzymes. The Michaelis constant is assumed to range from 10×10^{-6} to 100×10^{-6} mol liter^{-1} (8, 24). These values are higher than the molar oxygen concentrations at 0.4 and 1% DOT achieved in our experiments. The data are only rough estimates, since the Michaelis constant normally is determined with isolated enzymes. In the experiments described above, intact whole cells of microbial populations were used.

With regard to the estimation of K_m values in sediment suspensions containing hydrocarbons, it is important to know whether oxygen is accumulated in the hydrophobic micromilieu of the cells and in the hydrocarbon coat of the sediment particles at concentrations high enough to affect oxygenase reactions. This question and the question of whether the oxygenases are fully active with oxygen concentrations below their determined K_m need further investigation to help us understand degradation processes in sediments with low oxygen concentrations.

The experiments described here revealed that aerobic hexadecane biodegradation proceeds unaffected when the oxygen tension is lowered to 5% of the saturation concentration. Even if the DOT is lowered to 1% of saturation, the influence is negligible; a further diminution to 0.4% DOT alters the degradation kinetics but does not alter the degradation rate. These results cast new light on the conditions to be maintained during sediment remediation. It is significant that these results were achieved with the model substance hexadecane and not with petrochemical products or crude oil.

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REFERENCES

1. **Aeckersberg, F., F. Bak, and F. Widdel.** 1991. Anaerobic oxidation of saturated hydrocarbons to CO_2 by a new type of sulfate-reducing bacterium. Arch. Microbiol. **155**:5–14.
2. **Andersen, F. O., and W. Helder.** 1987. Comparison of oxygen microgradients, oxygen flux rates and electron transport system activity in coastal marine sediments. Marine Ecol. Prog. Ser. **37**:259–264.
3. **Atlas, R. M.** 1981. Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol. Rev. **45**:180–209.
4. **Bauer, J. E., and D. G. Capone.** 1985. Degradation and mineralization of the polycyclic aromatic hydrocarbons anthracene and naphthalene in intertidal marine sediment. Appl. Environ. Microbiol. **50**:81–90.
5. **Blanch, H. W., and A. Einsele.** 1973. The kinetics of yeast growth on pure hydrocarbons. Biotechnol. Bioeng. **25**:861–877.
6. **Bossert, I., and R. Bartha.** 1984. The fate of petroleum in soil ecosystems, p. 455–476. *In* R. M. Atlas (ed.), Petroleum microbiology—1984. Macmillan Publishing Co., New York.
7. **Boulton, C. A., and C. Ratledge.** 1984. The physiology of hydrocarbon-utilizing microorganisms. Top. Enzyme Ferment. Biotechnol. **9**:11–77.
8. **Boyer, P. D. (ed.).** 1975. The enzymes, vol. 12. Academic Press, Inc., New York.
9. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
10. **Bühler, M., and J. Schindler.** 1984. Aliphatic hydrocarbons, p. 329–385. *In* H. J. Rehm and G. Reed (ed.), Biotechnology—a comprehensive treatise in 8 volumes, vol. 6a, Biotransformation. Verlag Chemie, Weinheim, Germany.
11. **Dalyan, U., H. Harder, and T. Höpner.** 1990. Hydrocarbon biodegradation in sediments and soils. A systematic examination of physical and chemical conditions. II. pH values. Erdoel Kohle Erdgas Petrochem. **43(9)**:337–342.
12. **Dalyan, U., H. Harder, and T. Höpner.** 1990. Hydrocarbon biodegradation in sediments and soils. A systematic examination of physical and chemical conditions. III. Temperature. Erdoel Kohle Erdgas Petrochem. **43(11)**:435–437.
13. **Deutsche Sammlung von Mikroorganismen.** 1983. Catalogue of strains. Gesellschaft für Biotechnologische Forschung mbH, 3rd ed. Deutsche Sammlung für Mikroorganismen, Braunschweig, Germany.
14. **Findlay, R. H., G. M. King, and L. Watling.** 1989. Efficacy of phospholipid analysis in determining microbial biomass in sediments. Appl. Environ. Microbiol. **55**:2888–2893.
15. **Fricke, B., R. Bergmann, H. Sorger, and H. Aurich.** 1982. Optimierung von Kulturbedingungen für *Acinetobacter calco-*

aceticus beim Wachstum auf n-Alkanen in einem Laborfermenter. Z. Allg. Mikrobiol. **22(6)**:365–372.
16. **Gibbs, C. F.** 1975. Quantitative studies on marine biodegradation of oil. I. Nutrient limitation at 14°C. Proc. R. Soc. London B Biol. Sci. **188**:61–82.
17. **Hambrick, G. A., R. D. De Laune, and W. H. Patrick.** 1980. Effect of estuarine sediment pH and oxidation-reduction potential on microbial hydrocarbon degradation. Appl. Environ. Microbiol. **40**:365–369.
18. **Harder, H., B. Kürzel-Seidel, and T. Höpner.** 1991. Hydrocarbon degradation in sediments and soils. A systematic examination of physical and chemical conditions. IV. Special aspects of nutrient demand. Erdoel Kohle Erdgas Petrochem. **44(2)**:59–62.
19. **Höpner, T., H. Harder, K. Kiesewetter, U. Dalyan, I. Kutsche-Schmietenknop, and B. Teigelkamp.** 1989. Biochemical aspects of hydrocarbon biodegradation in sediments and soils, p. 251–271. *In* Z. Gerstel, Y. Chen, U. Mingelgrin, and B. Yaron (ed.), Toxic organic chemicals in porous media, ecological studies 73. Springer-Verlag KG, Berlin.
20. **Jorgensen, B. B., and N. P. Revsbech.** 1985. Diffusive boundary layers and the oxygen uptake of sediments and detritus. Limnol. Oceanogr. **30**:111–122.
21. **Kawataba, K., and K. Nakagawa.** 1989. Determination of microbial cell concentration based on total cellular C analysis. J. Microbiol. Methods **9**:337–344.
22. **Leahy, L. G., and R. Colwell.** 1990. Microbial degradation of hydrocarbons in the environment. Microbiol. Rev. **54**:305–315.
23. **Levring, T.** 1946. Some culture experiments with Ulva and artificial sea water. Kungliga Fysiografiska Sällskapets Lund förhandlingar **16(7)**:45–56.
24. **Lidstrom, M. E. (ed.).** 1990. Methods in enzymology, vol. 188. Hydrocarbons and methylotrophy. Academic Press, Inc., San Diego, Calif.
25. **MacDonald, R. M.** 1986. Sampling soil microfloras: problems in estimating concentration and activity of suspensions of mixed populations of soil microorganisms. Soil Biol. Biochem. **18(4)**: 411–416.
26. **Michaelsen, M., R. Hulsch, D. Haas, K. Koprek, B. Brunies, T. Höpner, and L. Berthe-Corti.** 1990. Influences on kinetics and carbon budget during hydrocarbon biodegradation in wadden sediment. Dechema Biotechnology Conference 4. VCH, Weinheim, Germany.
27. **Mihelcic, J. R., and R. G. Luthy.** 1988. Degradation of polycyclic aromatic hydrocarbon compounds under various redox conditions in soil-water systems. Appl. Environ. Microbiol. **54**:1182–1187.
28. **Mille, G., M. Mulyono, T. El Jammal, and J.-C. Bertrand.** 1988. Effects of oxygen on hydrocarbon degradation studies in vitro in surficial sediments. Estuarine Coastal Shelf Sci. **27**:283–295.
29. **Moriarty, D. J. W.** 1980. Measurement of bacterial biomass in sandy sediments, p. 131–139. *In* P. A. Trudinger (ed.), Biogeochemistry in ancient and modern environments. Springer, Berlin.
30. **Oberbremer, A., and R. Müller-Hurtig.** 1989. Aerobic stepwise hydrocarbon degradation and formation of biosurfactants by an original soil population in a stirred reactor. Appl. Microbiol. Biotechnol. **31**:582–586.
31. **Porter, K. G., and Y. S. Feig.** 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. **25**:943–948.
32. **Ratledge, C.** 1978. Degradation of aliphatic hydrocarbons, p. 1–46. *In* R. J. Watkinson (ed.), Developments in biodegradation of hydrocarbons, vol. 1. Applied Science Ltd., London.
33. **Rehm, H. J., and I. Reiff.** 1981. Mechanism and occurrence of microbial oxidation of long chain alkanes. Adv. Biochem. Eng. **19**:175–215.
34. **Schlegel, H. G.** 1985. Allgemeine Mikrobiologie, 6th ed. Thieme Verlag, Stuttgart, Germany.
35. **Singer, M. E., and W. R. Finnerty.** 1984. Microbial metabolism of straight-chain and branched alkanes, p. 1–47. *In* R. M. Atlas (ed.), Petroleum microbiology—1984. Macmillan Publishing Co., New York.
36. **Velji, M. I., and L. J. Albright.** 1986. Microscopic enumeration of attached marine bacteria of sea water, marine sediment, fecal matter, and kelp blade samples following pyrophosphate and ultrasound treatments. Can. J. Microbiol. **32**:121–126.
37. **Ward, D. M., and T. D. Brock.** 1978. Anaerobic metabolism of hexadecane in sediments. Geomicrobiol. J. **1**:1–9.